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Source: Avian Diseases, 47(s3):1091-1095. 2003.
Published By: American Association of Avian Pathologists
DOI: 10.1637/0005-2086-47.s3.1091
URL: http://www.bioone.org/doi/full/10.1637/0005-2086-47.s3.1091

AVIAN DISEASES 47:1091–1095, 2003

The Effect of Various Disinfectants on Detection of Avian Influenza Virus by Real Time RT-PCR

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Received November 4, 2002

SUMMARY. An avian influenza (AI) real time reverse transcriptase-polymerase chain reaction (RRT-PCR) test was previously shown to be a rapid and sensitive method to identify AI virus-infected birds in live-bird markets (LBMs). The test can also be used to identify avian influenza virus (AIV) from environmental samples. Consequently, the use of RRT-PCR was being considered as a component of the influenza eradication program in the LBMs to assure that each market was properly cleaned and disinfected before allowing the markets to be restocked. However, the RRT-PCR test cannot differentiate between live and inactivated virus, particularly in environmental samples where the RRT-PCR test potentially could amplify virus that had been inactivated by commonly used disinfectants, resulting in a false positive test result. To determine whether this is a valid concern, a study was conducted in three New Jersey LBMs that were previously shown to be positive for the H7N2 AIV. Environmental samples were collected from all three markets following thorough cleaning and disinfection with a phenolic disinfectant. Influenza virus RNA was detected in at least one environmental sample from two of the three markets when tested by RRT-PCR; however, all samples were negative by virus isolation using the standard egg inoculation procedure. As a result of these findings, laboratory experiments were designed to evaluate several commonly used disinfectants for their ability to inactivate influenza as well as disrupt the RNA so that it could not be detected by the RRT-PCR test. Five disinfectants were tested: phenolic disinfectants (Tek-trol and one-stroke environ), a quaternary ammonia compound (Lysol no-rinse sanitizer), a peroxygen compound (Virkon-S), and sodium hypochlorite (household bleach). All five disinfectants were effective at inactivating AIV at the recommended concentrations, but AIV RNA in samples inactivated with phenolic and quaternary ammonia compounds could still be detected by RRT-PCR. The peroxygen and chlorine compounds were effective at some concentrations for both inactivating virus and preventing amplification by RRT-PCR. Therefore, the RRT-PCR test can potentially be used to assure proper cleaning and disinfection when certain disinfectants are used.

RESUMEN. Efecto de varios desinfectantes en la detección del virus de influenza aviar mediante la prueba de la transcriptasa reversa-reacción en cadena por la polimerasa en tiempo real.

Se ha demostrado que la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real para el virus de influenza aviar es un método rápido y sensible para la identificación de aves infectadas con el virus de influenza aviar en centros de mercadeo de aves vivas. La prueba puede ser empleada igualmente para identificar el virus de influenza aviar a partir de muestras del medio ambiente. Por lo tanto, el uso de esta prueba ha sido considerado como componente del programa de erradicación de la influenza en centros de mercadeo de aves vivas con el fin de asegurar que cada centro de mercadeo ha sido limpiado y desinfectado

This proceedings manuscript documents an oral presentation given in the Session on Late Breaking Issues at the Fifth International Symposium on Avian Influenza, April 14–17, 2002, The University of Georgia, Athens, GA.

adecuadamente antes de permitir que estos centros sean abastecidos nuevamente. Sin embargo, la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real no puede diferenciar entre los virus vivos e inactivados, en especial en muestras del medio ambiente en las cuales esta prueba podría potencialmente amplificar virus que han sido inactivados por medio de desinfectantes empleados corrientemente, resultando en resultados positivos falsos. Con el fin de determinar la validez de esta preocupación, se realizó un estudio en tres centros de mercadeo de aves vivas en Nueva Jersey que habían sido positivos anteriormente al virus de influenza aviar H7N2. Se tomaron muestras del medio ambiente de los tres centros de mercadeo seguido de una limpieza y desinfección minuciosas con un desinfectante a base de fenol. Se detectó el ARN del virus de influenza aviar en por lo menos una muestra de medio ambiente en dos de los tres centros de mercadeo mediante la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real. Sin embargo, la totalidad de las muestras fueron negativas por aislamiento viral al emplear la técnica estándar de inoculación en huevos embrionados. Como resultado de estos hallazgos, se diseñaron experimentos de laboratorio para evaluar la capacidad de varios desinfectantes empleados corrientemente para inactivar el virus de influenza y alterar su ARN de tal manera que no puedan ser detectados mediante la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real. Se ensayaron 5 desinfectantes: desinfectantes a base de fenol (Tek-trol y One-Stroke Environ), un compuesto de amonio cuaternario (Lysol No-rinse Sanitizer), un compuesto de peróxido de hidrógeno (Virkon-S) e hipoclorito de sodio (blanqueador doméstico). Todos los desinfectantes fueron efectivos en la inactivación del virus de influenza aviar a las concentraciones recomendadas, sin embargo, el ARN del virus de influenza aviar en las muestras inactivadas con los compuestos fenólicos y de amonio cuaternario fue detectado mediante la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real. Los compuestos de peróxido de hidrógeno y clorinados fueron efectivos a algunas concentraciones para la inactivación del virus y la prevención de la amplificación del ARN mediante la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real. Por lo tanto, la prueba de la transcriptasa reversa – reacción en cadena por la polimerasa en tiempo real puede ser potencialmente empleada para asegurar una limpieza y desinfección adecuadas cuando son empleados ciertos desinfectantes.

Key words: avian influenza, chlorine, disinfectants, inactivation, phenolics, quartenary ammonia, real time RT-PCR

Abbreviations: AI = avian influenza; AIV = avian influenza virus; BHI = brain heart infusion; LBM = live-bird market; RRT-PCR = real time reverse transcriptase-polymerase chain reaction; SEPRL = Southeast Poultry Research Laboratory

Several diagnostic tests are available for the direct detection of avian influenza virus (AIV) including virus isolation in embryonated chicken eggs (12), real time reverse transcriptase-polymerase chain reaction (RRT-PCR) (11), and antigen capture immunoassays (10). All three tests can be valuable tools for detection of avian influenza (AI) in eradication programs, but all three have particular advantages and disadvantages. Virus isolation remains the gold standard, but RRT-PCR is being increasingly used because it has similar sensitivity to virus isolation and the results can be obtained much faster. One major difference between virus isolation and RRT-PCR is that virus isolation detects only live virus, but since RRT-PCR detects RNA, it potentially can detect both live and inactivated virus. The ability to distinguish between live and inactivated virus can be important for certain applications. For example, when you want to ensure that a premise has been properly cleaned and disinfected after it was depopulated of virus infected birds, sampling of the environment can be done, but virus isolation would be the diagnostic choice to ensure you are detecting live and not inactivated virus.

The use of disinfectants has been an integral component for infectious disease control programs, but the appropriate disinfectant needs to be selected based on the susceptibility of the target virus. Avian influenza virus is an enveloped virus with a segmented RNA genome, and it is grouped in the category of viruses that are among the easiest to inactivate (9). Influenza viruses can be inactivated by all the major classes of disinfectant if used properly (9). Influenza viruses are also susceptible to heat inactivation at modest levels, for example 57°C for 10 min has been shown to completely inactivate an avian influenza virus suspended in egg albumen (4).

Many different classes of disinfectants are commercially available, and each has a different mechanism for inactivating infectious disease agents. Common mechanisms of inactivation by disinfectants include denaturation of surface proteins, thus preventing viral attachment, and damaging viral nucleic acid thereby preventing virus replication. Phenolics, quaternary ammonium compounds, and alcohols act primarily by denaturing protein or lipid structures, preventing proper attachment and entry into the host cell. Halogens (chlorine) and peroxygen disinfectants are considered oxidants and can affect proteins, lipids, and nucleic acid (1,3). Other classes of disinfectants can have different modes of action, but the denaturants and oxidant group of disinfectants are most commonly used in veterinary medicine for inactivation of viruses on surfaces such as cages, floors, and feed trays.

For influenza viruses, no published reports were found comparing a disinfectant's ability to inactivate viruses as well as degrade the nucleic acid to prevent its detection by RT-PCR. Therefore, the purpose of this study was first to determine whether RRT-PCR is a suitable method for identifying whether a live-bird market still harbors residual infectious influenza virus after a thorough cleaning and disinfection, and second to evaluate commonly used disinfectants for their ability to both inactivate influenza viruses and disrupt the viral RNA so that they could not be detected by RRT-PCR.

MATERIAL AND METHODS

Evaluation of infected markets. Three live-bird markets, known to be positive for the H7N2 AIV based on a recent epidemiological survey, were selected to participate in a study to evaluate the use of RRT-PCR as a rapid method of determining whether a market was free of AIV after a thorough cleaning and disinfection. All three markets were cleaned and disinfected under the direct supervision of personnel from the New Jersey Department of Agriculture, Division of Animal Health. A phenolic based disinfectant (Tektrol, Bio-Tek Industries, Inc., Atlanta, GA), was used in all three markets at a dilution of 1/256. The following day multiple environmental swabs were taken throughout the market including cages, feed and water trays, drains, equipment, etc., and pools of five swabs, equaling one sample, were placed in 2-ml aliquots of brain heart infusion (BHI) broth as a transport media. A total of 30 pooled samples (150 swabs) were taken and placed on ice and sent to the Southeast Poultry Research Laboratory (SEPRL) by overnight courier service. At SEPRL 525 μl of the total sample was removed for RNA isolation with the Rneasy kit (Qiagen, Valencia, CA), and RRT-PCR was performed as previously described (11). The remainder of the sample was repackaged on ice packs and sent to the National Veterinary Services Laboratories by overnight courier service for virus isolation as previously described (11).

***In vitro* evaluation of disinfectants.** Five commonly used disinfectants were evaluated for efficacy in inactivating AIV and for their ability to disrupt AIV RNA so that it could not be detected by RRT-PCR. The disinfectants selected included two phenolic disinfectants, Tektrol and One-stroke Environ (Steris Corporation, St. Louis, MO); a quaternary ammonia compound, Lysol no-rinse sanitizer (Reckitt and Colman, Inc., Wayne, NJ); a chlorine compound, household bleach (sodium hypochlorite); and a peroxygen compound, Virkon S (Antec International, Sudbury, Suffolk, U.K.). Each disinfectant was diluted using single distilled water with the initial v/v dilution being 1/10 for bleach, 1/100 for Tektrol, One-stroke Environ, and Lysol no-rinse, and a 1% w/v concentration of Virkon S. Further dilutions of the working stocks of disinfectants were prepared in BHI broth. For all experiments, infectious virus in allantoic fluid containing either A/TK/WI/68 (H5N9) or A/TK/OR/71 (H7N3) was diluted 1/10 in BHI broth and 0.5 ml quantities were mixed with equal quantity of diluted disinfectant. Each mixture of virus/disinfectant was incubated for either 10 min or 1 hr at room temperature. Following incubation, the virus/disinfectant mixtures were either promptly processed for RNA extraction or inoculated into embryonating chicken eggs. An aliquot of 0.5 ml was used for RNA extraction, and the remaining 0.5 ml was inoculated into three eggs. However, samples at the highest concentration of disinfectant were only processed for RNA and not inoculated into embryonated chicken eggs because of the concern for toxicity to the embryo. Extracted RNA was tested by the RRT-PCR type A specific influenza test targeted to the influenza matrix gene as described earlier (11). Inoculated eggs were candled daily for 7 days, and any deaths during the first day were discarded as nonspecific deaths.

RESULTS AND DISCUSSION

Three randomly selected live-bird markets from New Jersey that were previously identified as having birds infected with AI H7N2 were depopulated of birds through normal commerce, and the premises were thoroughly cleaned and disinfected using Tektrol disinfectant. Personnel from the New Jersey Department of Agriculture supervised the cleaning and disinfection. The following day environmental swabs were taken from different parts of the market, and the samples were sent for RRT-PCR and virus

Table 1. The effect of 1 hr exposure of different disinfectants on the ability to inactivate influenza and prevent its detection by RRT-PCR.

Disinfectant/dilution	1/10	1/100	1/256	1/1000
Household bleach	NT[A]/(−)[B]	(−)[C]/(+)	(−)/(+)	
Environ one-stroke		NT/(+)	(−)/(+)	(−)/(+)
Tektrol		NT/(+)	(−)/(+)	(−)/(+)
Lysol no-rinse		NT/(+)	(−)/(+)	(+)/(+)
Virkon-S (fresh)		NT/(−)	(−)/(−)	(−)/(+)
Virkon-S (10 days old)		NT/(+)	(+)/(+)	(+)/(+)

[A]NT = not tested.
[B](−) negative for RT-PCR.
[C](−) negative by virus isolation.

isolation. No AIV was detected in any of the environmental samples when tested by virus isolation. However, AIV RNA was detected in multiple samples from two of the three markets. All but one of the samples were positive at a high cycle threshold, indicating only a small amount of virus was present in the sample. Most of the RRT-PCR matrix positive samples were also confirmed positive with the H7 subtype specific primer set, increasing the confidence in the original RRT-PCR test results. The positive samples included swabs from a feed bag, feed bins, crates, and floors.

Results of environmental testing in live-bird markets (LBMs) suggested that the phenolic disinfectant used in the market inactivated the AIV in the market, but residual inactivated virus could still be detected by RRT-PCR. This correlates well with the perceived mode of action for phenolic disinfectants, which is the inactivation of viruses by denaturation of surface proteins. Phenolic disinfectants by themselves likely cause little or no damage to nucleic acid, particularly since phenol is commonly used for extraction of nucleic acid from clinical samples. However, it was not clear from previous reports whether inactivation of influenza viruses by phenolic disinfectants resulted in the release of the viral RNA into the environment where it could be destroyed by ubiquitous RNAses. The results from the field study suggested that the inactivated virus remained intact and continued to protect the viral RNA.

Since different disinfectants have different modes of action for inactivation of viruses, additional laboratory studies were performed to examine the efficacy of five disinfectants to inactivate AIV and disrupt the RNA so that it would not be detected by RRT-PCR. Results for 1 hr incubation of mixtures of virus/disinfectant are presented in Table 1. All five disinfectants inactivated AIV when used at the manufacturer's recommended dilution rates (1/10 for household bleach; 1/256 for Tektrol, One stroke environ, and no-rinse Lysol; 1/100 for Virkon-S). Three of the disinfectants were also effective when tested at the 1/1000 dilution. For the Virkon-S, both a freshly prepared solution and a solution prepared 10 days previously were tested, but only the fresh solution inactivated the virus under the conditions tested. This is not unexpected since the manufacturer's recommendations are to discard the disinfectant 1 week after the dry powder is mixed into solution.

Results of the *in vitro* evaluation of disinfectants showed that three of the five disinfectants were unable to damage the RNA effectively to prevent detection by RRT-PCR. The exceptions were household bleach at 1/10 dilution and fresh Virkon-S at 1/100 and 1/256. The effective concentrations are within the recommended dilutions for both disinfectants. Neither the phenolic nor the quaternary ammonia compounds appeared to have any effect on the viral RNA.

Results observed in this report are similar to results achieved with disinfectants with other viral and bacterial systems. Three previous papers have demonstrated the ability of sodium hypochlorite or free chlorine to prevent RT-PCR detection in hepatitis C virus, poliovirus, and rotavirus, respectively (2,5,8). However, in the poliovirus model, it took three to six times longer for the free chlorine to prevent RRT-PCR detection than it did to inactivate the virus as detected by cell culture (5). This would suggest that the chlorine needs to disrupt a specific site for the RT-PCR to fail, but it can disrupt potentially any site of the virus and cause inactivation. This type of effect has been observed with studies with *Legionella pneumophila*, where the use of PCR with a small amplicon was much more resistant to PCR failure as compared to a large amplicon after treatment with chlorine disinfectant

(7). The use of H_2O_2 with rotaviruses had similar results with both viral inactivation and negative results on RT-PCR (8). Little work has been done with phenolic disinfectants, but phenol is a commonly used chemical to extract RNA and DNA from samples. In one study with bacteriophage F116 that was tested with several disinfectants, phenol had no affect on viral DNA, but glutaraldehyde and peracetic acid both degraded the viral RNA (6).

The results from both the field and *in vitro* studies presented here suggest that the phenolic disinfectants are effective for inactivating AIV, but they do not adversely affect the viral RNA to the point where it prevents detection by RRT-PCR. The five disinfectants used in this study were effective at inactivating AIV as measured by virus isolation. This is not surprising since influenza is classified in one of the easiest groups of viruses to be inactivated (9). The study, however, points out that different disinfectants inactivate virus by different methods, and only the chlorine compounds and peroxygen compounds tested in this study damaged the RNA such that it could not be detected by RRT-PCR.

One of the goals of the study was to determine whether RRT-PCR could be used as a way to ensure that markets that had AIV infected birds were properly cleaned and disinfected before repopulation was allowed. The results clearly show that phenolic disinfectants will inactivate virus but not prevent its detection by RRT-PCR, and therefore RRT-PCR is not a suitable diagnostic test for environmental samples under these conditions. The quaternary ammonium compound tested, based on *in vitro* data, would also be inappropriate for this type of environmental testing. Chlorine and peroxygen compounds could potentially be used for environmental testing. However, the concentration required to inactivate the viruses with these compounds was lower than the concentration necessary to degrade the RNA enough not to be detected by RRT-PCR. Therefore, additional field testing would be required to determine the usefulness of RRT-PCR for environmental testing before repopulation.

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ACKNOWLEDGMENTS

The authors thank Dr. Chang-won Lee and Suzanne DeBlois for technical assistance.